neurotransmitter signaling. While protein targets for many drugs are well-characterized, drug effects on membrane lipids, which are responsible for regulating signaling, are less clear. We used small angle neutron scattering, a novel technique to study phase separation in small (100-30 nm) vesicles made from mixtures of lipids, to study the effect of antidepressants on model raft forming lipid mixtures. Using as our staring point the raft-mimicking lipid mixture: 1:1:1 DOPC:DPPC:Cholesterol, we systematically replaced cholesterol with the drug Escitalopram and its clinically-inactive isomer, Rcitalopram. Escitalopram and Rcitalopram differ only by their opposing chiralities; the former is an effective antidepressant drug while the latter, Rcitalopram, is not. There is a clear difference in the behavior of domain size and composition when cholesterol is replaced by both Escitalopram and Rcitalopram, however, these changes are similar. These results suggest that drugs like Escitalopram do affect the membrane environment, potentially facilitating drug action, but ultimately, Escitalopram's chirality is important for protein interaction.

**3139-Pos Board B294**

**Revealing the Relationship between Fibroblast Growth Factor Receptor-Like 1 (FGFRL1) and Free Zinc in Pancreatic Beta-Cells using Quantitative Fluorescence Microscopy**

**Pamuditha N. Silva**, Dawn M. Kilkenny, Jonathan V. Rocheleau.
University of Toronto, Toronto, ON, Canada.

Type 2 diabetes is characterized by a loss of normal pancreatic beta-cell function and mass. In beta-cells, tyrosine kinase Fibroblast Growth Factors Receptors (FGFRs) modulate insulin processing, fatty acid metabolism and cell survival. However, a deeper understanding of mechanisms that regulate function of these receptors will be necessary to use this pathway therapeutically. We have recently identified beta-cell expression of Fibroblast Growth Factor Receptor-like 1 (FGFRL1), a newly-identified member of the FGFR family. FGFRL1 shares the canonical extracellular domain of FGFRs but uniquely exhibits a short C-terminal histidine-rich zinc-binding domain rather than intracellular catalytic kinase domains. Zinc is a second messenger normally found at picomolar concentration in the cytosol that mediates phosphatase activity to regulate Mitogen-Activated Protein Kinase (MAPK) signaling. We determined that FGFRL1 co-localizes with insulin secretory granules where zinc accumulates at micromolar concentrations. We therefore postulate that FGFRL1 alters beta-cell MAPK signaling by chelating zinc and regulating its intracellular concentration. To measure intercellular zinc, we imaged living murine beta-cells expressing full-length and truncated fluorescent protein variants of FGFRL1 co-labeled with cell-permeable zinc indicators FluoZin-3 and RhodZin-3. Our data confirm that FGFRL1 reduces free intracellular zinc via the unique histidine-rich region. We further show that zinc induces FGFRL1 receptor dimerization at the cell membrane using homo-fluorescence resonance energy transfer (Homo-FRET) imaging. Dimerization of FGFRL1 and association with insulin secretory granules suggest receptor activity is tightly regulated by zinc and likely associated with glucose-stimulated insulin secretion. Elucidating novel signaling mechanisms that regulate FGFR-activity in beta-cells will improve our understanding of how this pathway can be used therapeutically to treat diabetes.

**3140-Pos Board B295**

**Cells must Accumulate Interleukin-4 Receptor Subunits within Cortical Signaling Endosomes to Drive Complex Formation and Signal Transduction**

Kristina Kurgonaite[1], Hetvi Gandhi[2], Remigiusz Worch[3], Benjamin Obermann[2], Martin Hintersteiner[4], Sophie Pautot[1], Sigrun R. Hofmann[5], Christian Bökel[1], Petra Schwille[6], **Thomas Weidemann**[6].
[1]Technical University Dresden, Center for Regenerative Therapies Dresden, Germany, [2]Technical University Dresden, BIOTEC, Germany, [3]Polish Academy of Sciences, Warsaw, Poland, [4]Novartis Institutes for BioMedical Research, Basel, Switzerland, [5]Technical University Dresden, University Hospital Carl Gustav Carus, Germany, [6]Max Planck Institute of Biochemistry, Munich, Germany.

The cytokines Interleukin-4 (IL-4) and IL-13 provide cues for many important immune functions and their upregulation is associated with disorders like allergy and asthma. Signaling requires ligand mediated dimerization of single-pass transmembrane receptors. We recently established epithelial HEK293T cells as a model to characterize ectopically expressed IL-4R subunits with single- and dual-color fluorescence correlation spectroscopy [1]. Here we report an improved experimental setup employing hexahistidine specific dyes and demonstrate for the first time ligand-induced IL-4R complex formation in a native plasma membrane. Furthermore, we quantified the two-dimensional affinity constants for all three combinations of receptor dimers with 120 (IL-4:IL-4Rα/IL-13Rα1), 510 (IL-13:IL-13Rα1/IL-4Rα), and 825 (IL-4:IL-4Rα/IL-2Rγ) receptor molecules per $\mu m^2$. However, considering physiological surface expression levels of several 100-1000 receptor molecules per cell, such low affinities challenge the traditional view that signaling productive complexes self-assemble in significant numbers in the plasma membrane. Instead, we mount several lines of evidence that signal transduction requires the accumulation of the receptor subunits within a novel class of early endosomes. These cortical signaling endosomes are stably anchored within the actin cortex just beneath the plasma membrane and carry markers of both the early sorting (EEA1, Rab5) and recycling compartments (Rab11). The IL-4R subunits show Rac1/Pak-dependent trafficking from the surface into these endosomes with a time constant of 6-9 min. Using fluorescence lifetime imaging / Förster resonance energy transfer (FLIM/FRET) microscopy, we could demonstrate ligand-dependent complex formation within the cortical signaling endosomes. Importantly, specific inhibition of the endocytosis machinery with drugs abrogates both receptor trafficking and phosphorylation of the downstream signal transducer STAT6. In summary, our findings suggest a unique thermodynamic function for endocytosis upstream of JAK/STAT pathway activation.

[1] Weidemann, T., *et al.* (2011) *Biophys. J.***101:** 2360-69.

**3141-Pos Board B296**

**The Neck Region Regulates Spatiotemporal Organization and Virus-Binding Capability of the Pathogen Recognition Receptor DC-Sign**

Juan A. Torreno-Pina[1], Carlo Manzo[1], Carl G. Figdor[2], Alessandra Cambi[2], **Maria F. Garcia-Parajo**[1,3].
[1]ICFO-Institute of Photonic Sciences, Castelldefels, Barcelona, Spain, [2]Nijmegen Centre for Molecular Life Sciences, Nijmegen, Netherlands, [3]ICREA-Institució Catalana de Recerca i Estudis Avançats, Barcelona, Spain.

Receptor nanoclustering *prior* to ligand activation is rapidly emerging as an essential feature common to most cell membranes. Yet, the mechanisms governing this distinct spatial patterning and functional role are still poorly understood. We used a combination of biochemical and advanced biophysical techniques, including optical superresolution and single particle tracking, to investigate the spatiotemporal organization of DC-SIGN, a pathogen recognition receptor that homo-oligomerizes in-vitro. We found an intrinsic nanoclustering capacity of DC-SIGN (*ca.* 180 nm in size) far beyond basal tetramerization, which strictly depended on its molecular structure. DC-SIGN nanoclusters exhibited Brownian diffusion on the cell membrane with values of the order of $10^{-2}$ $\mu m^2/s$. Truncation of the neck region, known to abrogate tetramerization, significantly reduced nanoclustering and concomitantly increased lateral diffusion. Importantly, DC-SIGN nanocluster dissolution compromised DC-SIGN binding to nanoscale size pathogens. As such, our results underscore a direct relationship between spatial nanopatterning, driven by intermolecular interactions between the neck regions, and receptor diffusion to provide DC-SIGN with the exquisite ability to dock pathogens at the virus length-scale. We suggest that protein-protein interactions facilitated by structural molecular motifs might represent a so far underestimated but general mechanism to pre-organize receptors on the cell membrane for efficient action under stimulated conditions.

**3142-Pos Board B297**

**Lysophosphatidic Acid Signalling in Red Blood Cells**

**Lars Kaestner**[1], Jue Wang[1], Lisa Wagner-Britz[2], Anna Bogdanova[3], Kathrina Wiesen[1], Qinghai Tian[1], Ingolf Bernhardt[2], Stephan Philipp[1], Marc Freichel[4], Veit Flockerzi[1], Peter Lipp[1].
[1]Saarland University, Homburg/Saar, Germany, [2]Saarland University, Saarbrücken, Germany, [3]University of Zürich, Zürich, Switzerland, [4]Uniersity of Heidelberg, Heidelberg, Germany.

Red blood cells are among the most intensively studied cells in natural history, elucidating numerous principles and ground-breaking knowledge in cell biology. Morphologically, red blood cells are largely homogeneous, and most of the functional studies have been performed on large populations of cells, masking putative cellular variations. We studied human and mouse red blood cells by live-cell video imaging, which allowed single cells to be followed over time. In particular we analysed functional responses to hormonal stimulation with lysophosphatidic acid, a signalling molecule occurring in blood plasma, with the calcium sensor Fluo-4. Additionally, we developed an approach for analysing the calcium responses of red blood cells that allowed the quantitative characterisation of single-cell signals. In red blood cells, the lysophosphatidic acid-induced calcium influx showed substantial diversity in both kinetics and amplitude. Also the age-classification was determined for each particular red blood cell and consecutively analysed. While reticulocytes lack a calcium response to lysophosphatidic acid stimulation, old red blood cells approaching clearance generated robust lysophosphatidic acid-induced signals, which still displayed broad heterogeneity. We revealed the intracellular signalling from the lysophosphatidic acid receptors to the calcium channels.

The functional diversity of red blood cells needs to be taken into account in future studies, which will increasingly require single-cell analysis approaches. The identified lysophosphatidic acid signalling cascade provides as a multicomponent system the potential for delays and gains and such provokes a tremendous variability. Heterogeneity in red blood cell responses is important for the basic understanding of red blood cell signalling and their contribution to numerous diseases, especially with respect to calcium influx and the associated pro-thrombotic activity.

**3143-Pos Board B298**

**The Molecular Basis of Substrate Recognition by the E3 Ubiquitin Ligase Pellino**

**Yu-San Huoh**, Kathryn M. Ferguson.
University of Pennsylvania, Philadelphia, PA, USA.

The Pellino proteins are one of the several families of E3 ubiquitin ligases that direct ubiquitination events immediately following Toll and interleukin-1 receptor (TIR) activation. Polyubiquitination of known Pellino substrates, such as the interleukin-1 associated kinase (IRAK1), is a necessary step in mediating downstream signaling events that are responsible for eliciting a proper immune response. To elucidate Pellino's role in TIR signaling, we are investigating the molecular basis of Pellino substrate specificity. We previously determined the X-ray crystal structure of the human Pellino2 substrate recognition domain and found that it contains a non-canonical example of a well-characterized phosphothreonine (pT)-binding domain, the forkhead-associated (FHA) domain. In an attempt to determine the specific substrate-binding motif of Pellino2, we identified an IRAK1 truncation variant (aa 1-197, IRAK1-197) that interacts with Pellino2 in a phosphorylation dependent manner. Substitution of each threonine in IRAK1-197 with alanine identified T141 as the critical phosphorylated threonine on IRAK1-197 that Pellino2 specifically recognizes. A synthetic phosphopeptide corresponding to the sequence centered on T141 of IRAK1 (pT141 peptide) binds to Pellino2 with a Kd value in the 1 uM range; this data was assessed in a fluorescence polarization binding assay, and independently verified using isothermal titration calorimetry. Binding analyses of other mammalian Pellino isoforms (Pellino 1, 3A, and 3B) to the pT141 peptide reveals differences in binding affinities and specificities. These differences are hard to reconcile due to the high degree of sequence identity among the Pellino isoforms, and cannot be readily explained by the Pellino2 FHA domain crystal structure. Thus, to further explore the molecular basis of these differences, we are working towards determining the X-ray crystal structures of the other Pellino isoforms.

**3144-Pos Board B299**

**Structural and Thermodynamic Insights into Bacterial Outer Membrane Lipid Signaling by the Innate Immune System**

**Teresa Paramo**, Susana Tomasio, Peter J. Bond.
University of Cambridge, Cambridge, United Kingdom.

Lipopolysaccharide (LPS) from bacterial outer membranes is a potent early indicator of microbial infection and the primary inducer of fatal septic shock syndrome. Its recognition by the immune system is carried out by Toll-like receptor 4 (TLR4) when associated with its co-receptor MD-2, an immunoglobulin-like protein. MD-2 adopts a characteristic ''beta-cup'' fold with a large hydrophobic cavity, and is able to bind a variety of lipid species. Subtle alterations in the structure of LPS derivatives can profoundly alter the resultant immunological response, hampering the rational design of TLR4 immunomodulators. To unravel the associated structure-activity relationships, we have performed long-timescale, all-atom molecular dynamics simulations and free-energy calculations of the isolated MD-2 co-receptor and the entire signaling-active receptor complex in the presence of a variety of LPS species, as well as an LPS membrane. Unbiased simulations revealed that the MD-2 cavity is highly conformationally flexible, identifying spontaneous switching between active signalling-competent and inactivated states dependent upon the presence of different ligands, leading us to propose a conserved receptor activation mechanism. To gain insights into the thermodynamic determinants of endotoxin recognition, extensive umbrella sampling has been applied to estimate the potential of mean force (PMF) for the binding of LPS molecules to MD-2 co-receptor. Strikingly, stronger binding to signalling-inactivate MD-2 were observed for antagonists, and conversely, stronger binding to active MD-2 for agonists. Comparison of this data to the first ever PMF calculated for extraction of LPS from a model of the bacterial outer membrane has revealed how MD-2 creates a ''membrane-like'' environment within its protein cavity, providing a mechanism for sensitive LPS recognition by the innate immune system.

**3145-Pos Board B300**

**Molecular Mechanisms of Glutamate Receptor Activation and Regulation**

**John Belcher**, Albert Lau.
Johns Hopkins University, Baltimore, MD, USA.

Neuronal signaling is based on the release and detection of glutamate within the synaptic cleft. Regulation of this signaling has implications in both learning and memory, while dysfunction is implicated in a variety of neurological disorders, including schizophrenia and depression. Glutamate in the synaptic cleft is detected by ionotropic glutamate receptors (iGluRs), which are ligand-gated ion channels. NMDA receptors (NMDARs) are a class of iGluRs that require the binding of both glycine and glutamate for receptor activation. We are concerned with the molecular mechanisms that govern the activity of NMDARs, both in terms of activation and regulation. Here, the free energy landscapes governing large-scale conformational transitions in the isolated ligand-binding domains (LDBs) of the NMDAR subunits GluN1, GluN2A, and GluN3A are computed for both apo and holo forms using umbrella sampling simulations. Agonist insertion shifts the bias to closed conformations to various degrees for the different LBDs. We compare the structural elements that determine the stability of the open and closed states of NMDARs with those in a bacterial homologue GluR0. In addition we present structural data examining the interaction between a subclass of AMPA receptors (AMPARs), another class of iGluRs, and isolated elements of protein 4.1, a chaperone that regulates AMPAR insertion into the plasma membrane.

**3146-Pos Board B301**

**Non-Genomic Progesterone Signalling in Human Sperm**

**Christoph Brenker**[1], Astrid Müller[1], Christian Trötschel[2], U.B. Kaupp[1], Timo Strünker[1].
[1]Center of Advanced European Studies and Research, Bonn, Germany, [2]Ruhr-University, Bochum, Germany.

In human sperm, CatSper (cation channel of sperm) $Ca^{2+}$ channels control the intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]i$) and, thereby, the swimming behaviour. By patch-clamp recordings from human sperm and kinetic $Ca^{2+}$ fluorimetry, we have shown that CatSper is directly activated by the female sex hormone progesterone; cells surrounding the oocyte release progesterone into the oviduct to assist sperm for fertilization. The rapid $Ca^{2+}$ influx evoked by progesterone has been implicated in sperm chemotaxis, hyperactivation, and acrosomal exocytosis. We studied progesterone-evoked voltage responses in human sperm with the kinetic stopped-flow technique, using electrochromic voltage-sensitive dyes. We show that progesterone evokes instantaneous changes in membrane voltage (Vm) governed by activation of CatSper and another ion channel present in human sperm. We elucidated the molecular identity of this channel by patch-clamp recordings from human sperm.

**3147-Pos Board B302**

**Protein-Protein-Protein Interactions in Membranes Measured by Triple Cross-Correlation of Confocal Images**

Max Anikovsky[1], **Nils O. Petersen**[2,3].
[1]University of Calgary, Calgary, AB, Canada, [2]University of Alberta, Edmonton, AB, Canada, [3]National Institute for Nanotechnology, Edmonton, AB, Canada.

**Protein-protein** interactions in binary complexes have been measured successfully and quantitatively by fluorescence correlation spectroscopy in solution (Elson, BJ 101, 2855-2870 (2011)) and image correlation spectroscopy on cell surfaces (Kolin and Wiseman, Cell Biochem Biophys 49, 141-164 (2007)). These tools fail, however, to provide information about ternary complexes i.e. about **protein-protein-protein** interactions. It has been known for some time that higher order moments or correlations contain the relevant information (Palmer and Thompson, BJ 52, 257-270 (1987); Heinze, Jahnz, and Schwille, BJ 86, 506-516 (2004)) but it is only recently that triple correlation functions of ternary complexes have been measured in solution (Ridgeway, Millar, and Williamson, J. Phys Chem B 116, 1908-1919 (2012) and PNAS 109, 13614-13619 (2012)). Our present work shows how complete and quantitative information can be obtained for ternary complexes of membrane proteins from three confocal images from each of three distinctly labeled protein species by employing a combination of image correlation spectroscopy, image cross-correlation spectroscopy, image triple auto-correlation spectroscopy and image triple cross-correlation spectroscopy.

**3148-Pos Board B303**

**Detailed Analysis of Phospholipase C-β Activity in Living Cells**

**Michael G. Leitner**, Oliver Dominik.
Institute for Physiology, Department of Neurophysiology, Philipps University Marburg, Marburg, Germany.